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13. ABSTRACT *(Maximum 200 Words)*

Galectin-3 is a β-galactoside-binding protein which regulates many biological processes including cell adhesion, migration, cell growth, tumor progression, metastasis and apoptosis. Although the exact function of galectin-3 in cancer development is unclear, galectin-3 expression is associated with neoplastic progression and metastatic potential. Since studies have suggested that tumor cell survival in microcirculation determines the metastatic outcome, we examined the effect of galectin-3 overexpression in human breast carcinoma cell survival using the liver ischemia/reperfusion metastasis model. While the majority of control cells died by hepatic ischemia/reoxygenation, nearly all of galectin-3 overexpressing cells survived. We showed that galectin-3 inhibits nitrogen free radical-mediated apoptosis, one of the major death pathways induced during hepatic ischemia/reperfusion. Galectin-3 inhibition of apoptosis involved protection of mitochondrial integrity, inhibition of cytochrome c release and caspase activation. Taking these results together with the previous observation that galectin-3 inhibits apoptosis induced by loss of cell adhesion, we propose that galectin-3 is a critical determinant for anchorage-independent and free radical-resistant cell survival during metastasis.

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Research for the period 1 Jul 99 - 30 Sep 00

(4) Introduction

Galectin-3 is a 30 kDa protein member of the β-galactoside-binding family that is highly expressed in various human tumor cells (1-5). In vitro, galectin-3 modulates a variety of biological processes such as cell adhesion, migration, cyst formation, secretion of cytokines, and pre-mRNA splicing (6-11). Although the exact function of galectin-3 in cancer development is unclear, its expression is associated with neoplastic progression and metastatic potential (1-6, 12, 13).

Recently, we as well as others showed that galectin-3 protects cells against apoptosis (14-17). Inflammatory cells from galectin-3 deficient mice were more prone to undergo apoptosis, re-emphasizing a galectin-3 role for apoptosis regulation (18).

During the funding period, we demontrated a role for galectin-3 in the regulation of genistein-mediated cellular responses. We show that genistein effectively induces apoptosis without detectable cell cycle arrest in BT549, a human breast epithelial cell line which does not express galectin-3 at a detectable level. In galectin-3 transfected BT549 cells, genistein induced cell cycle arrest at the G_2/M phase without apoptosis induction. Interestingly, genistein induces $p21^{WAF1/CIP1}$ expression in galectin-3 expressing BT549 cells, but not in control BT549 cells undergoing apoptosis. Our study suggests that galectin-3, at least in part, is a critical determinant for genistein-mediated cell cycle arrest and apoptosis, and genistein induction of $p21^{WAF1/CIP1}$ is associated with cell cycle arrest, but not required for apoptosis induction (Appendix 1, Carcinogenesis 21, 1941-1945, 2000).

During the funding period, we also investigated the role for galectin-3 in breast carcinoma cell metastasis. Tumor cell metastasis is a complex process involving motility, invasion, and cell growth and survival. It was observed that the ability of tumor cells to colonize the liver was shown to be proportional to the number of tumor cells surviving within the sinusoid (19, 20). As tumor cells arrest in the hepatic sinusoid and terminal portal venules, microscopic infarcts develop, followed by re-establishment of blood flow. Ischemia/reperfusion results in oxygen and nitrogen radical formation that is toxic to the majority of the arrested tumor cells (21, 22). However, the survival rate of highly metastatic human carcinoma cells is 10-fold higher than that of weakly metastatic cells during hepatic ischemia and reperfusion (21, 22). Presently, the molecular mechanisms by which metastatic tumor cells become resistant to oxygen and nitrogen radicals during hepatic ischemia/reperfusion remain unclear. To examine whether galectin-3 protects human breast carcinoma cells from death during hepatic ischemia-reperfusion, we used a mouse ischemic liver tissue/human breast tumor cell coculture system (see "Body of Report). We also investigated the effect of galectin-3 on nitric oxide-induced apoptosis, one of the major death pathways induced during hepatic ischemia-reperfusion.

(5) Body of Report

Methods

Cell culture

The human breast carcinoma cell line BT549 was obtained from Dr. E. W. Thompson, Vincent T. Lombardi Cancer Research Center, Georgetown University Medical Center (Washington, D. C.). Galectin-3 transfected BT549 cell clones were previously established by introducing an expression vector containing human galectin 3 cDNA into BT549 parental cells (15, 16). Galectin-3-transfected BT549 and neo-resistant control vector transfected BT549 cells are referred to as BT549Gal-3 and BT549neo, respectively. Cells on tissue culture dishes (Sarstedt, Newton, NC) were grown in DMEM/F-12 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.5 μg/ml fungizone in a 95% air and 5% CO_2 incubator at 37^0C.

Cell labeling with Rd-Dx and calcein AM

BT549Gal-3 and BT549neo cells were labeled with two fluorescing reagents, rhodamine B-isothiocyanate dextran (Rd-Dx; Sigma Chemical Co., St. Louis, MO), with maximum excitation/emission at 530/590 nm, and calcein AM, with maximum/emission at 485/530 nm. Rd-Dx was loaded into BT549 cells by electroporation (Cell-Porator, BRL, Gaithersburgh, MD). Cells ($5x10^6$), suspended in 20 mg/ml Rd-Dx in

phosphate-buffered saline (PBS) solution, received an electrical pulse (capacitance: 330 μF and 300 V; load resistance: high Ω; charge rate: fast). The Rd-Dx-loaded cells were incubated for 16 hr at 37°C in complete tissue culture medium, and then adherent cells were recovered by trypsin. The cells were suspended in PBS and incubated with calcein AM (Molecular Probes, Eugene, OR) at a final concentration of 4 μM for 30 min at 37°C.

Liver-BT549 co-culture

Athymic nude mice purchased from Harlan Sprague Dawley Inc. (Frederick, MD) were anesthetized with a general inhalant as previously described (22). A midline laparotomy incision was made under aseptic conditions to expose the liver. To induce ischemia, the portal vein and hepatic artery were clamped with a microvascular clamp for 3 min. The liver was excised and immediately placed in chilled DMEM/F-12 on ice and dissected into 1-3 mm fragments. The liver fragments were transferred into a 50-ml centrifuge tube and washed once by centrifugation at 400 x g for 5 min. The fragments were resuspended in 5 ml of the co-culture medium and loaded into a 55-ml Rotating Wall Vessel (RWV; Rotary Cell Culture System, Synthecon, Inc., Houston, TX). BT549 cells pre-labeled with Rd-Dx and calcein AM were then added to the RWV, and the 55-ml RWV chamber was completely filled with the liver fragment co-culture medium [a 1:1 mixture of HepatoZYME-SFM and DMEM/F-12 with 10% FBS and penicillin-streptomycin] to eliminate bubbles. An air pump was connected to the RWV for reoxygenation after liver ischemia. After co-culture, Rd-Dx and calcein AM-labeled cells were detected on a Nikon upright microscope (Nikon Microphot-FXL, Tokyo, Japan) equipped with epi-fluorescence using digital image capture by an 3CCD color video camera (Model DXC-930P, Sony, Tokyo, Japan).

Mitochondria staining

Cells were plated on a coverslip in a 6-well plate. After 24 hr of apoptosis induction, the cells were incubated with media containing 250 μM MitoTracker Red (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Cells were washed with PBS, fixed with 3.7% paraformaldehyde in PBS for 15 min 37°C. The coverslips were mounted onto glass plates using 0.1% phenylenediamine and 90% glycerol in PBS. Fluorescent staining of the mitochondrial membrane was examined with a Nikon Labophot microscope fitted with a digital video camera (Photometrics, Tucson, AZ).

Cytochrome c release

Cells were harvested at 0, 24, and 48 hr following treatment with 200 μM S-nitroso-N-acetylpenicillamine (SNAP), resuspended in ice-cold lysis buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5mM MgCl_2, 1 mM sodium EDTA, 1 mM sodium EGTA, 20 μl protease inhibitors (Sigma, MO), 4 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose, and incubated for 1 hr at 4°C. The lysates were passed through a 26 gauge syringe 15 times and then centrifuged at 10,000 rpm for 20 min at 4°C. The resulting supernatant was analyzed by immunoblot analysis using anti-cytochrome c antibody (ZYMED Laboratories Inc, CA) as previously described (23).

DEVDase activity

Cells were collected at 0, 24, 36, 48 and 60 hr after treatment with 200 μM S-nitroso-N-acetylpenicillamine (SNAP, Sigma Chemical Co., MO), and lysed in caspase lysis buffer [50mM Tris buffer (pH 7.5), 0.03% Nonidet and 1 mM DTT]. Nuclei were removed by low speed centrifugation(800g, 5 min), and the cytosolic fraction was incubated with 40 μM DEVD-amc, 10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT for 2 hr at 37°C. Fluromethylcoumarin fluorescence, released by DEVDase (caspase) activity, was measured using 380 nm excitation. A CCD device (Instapec IV; Oriel, Straford, CT) fitted with a monochromator was used to measure the fluorescence emission spectrum. The intensity at the optimum (~450 nm) was measured. DEVDase activity was normalized per microgram of protein determined by BCA protein assay kit (Pierce, IL).

Results

To study the role of galectin-3 in inhibiting human breast caricnoma cell death during liver ischemia/reperfusion, we cultured BT549Gal-3 or BT549neo cells in a rotating suspension culture system containing mouse liver fragments, a co-culture system previously shown to maintain the architecture and viability of the liver for at least 24 hr (22). Hepatic ischemia was induced by clamping the hepatic arterial and portal vein, and an air pump was connected to the rotating suspension culture system to mimic the *in vivo* reoxygenation after liver ischemia. To distinguish human breast carcinoma cells from mouse liver cells, the human cells were labeled with rhodamine B-isothiocyanate dextran (Rd-Dx). The previous study (24) demonstrated that rhodamine-conjugated dextran (Rd-Dx) is non-toxic, does not leach out of cell cytoplasm, and retains its fluorescence until diluted by cell division. To distinguish viable cells after the co-culture period, the human cells were also prelabeled with calcein AM, since it produces fluorescence signal only in live cells with ATP-dependent cytoplasmic esterase activity. As shown in Fig. 1, control mouse liver cells were not toxic to human breast carcinoma cells. When BT549neo cells were co-cultured with ischemic liver fragments, only 62% of the cells survived at 24 hr. In contrast, all of BT549Gal-3 cells survived after 24 hr co-culture with ischemic liver fragments. These results imply that galectin-3 expression in human breast carcinoma cells protects against cell death during during liver ischemia/reperfusion.

Previous studies showed that reactive nitrogen free radicals generated during liver ischemia/reperfusion are the major toxic molecules for the weakly metastatic colorectal cancer cells (22). Here, we examined whether the same molecular species are responsible for killing BT540neo cells. To this end, mice were pretreated by a tail vein injection with 20 mg of N^G-monomethyl-L-arginine (NMMA), a NO synthase inhibitor, for 30 min before ischemic treatment. The isolated liver fragments were co-cultured with BT549neo cells in the presence of 1 mM NMMA. The cell viability with NMMA treated ischemic liver fragments increased to the comparable levels of co-culture with control mouse liver fragments. These suggested that reactive nitrogen free radicals generated during hepatic liver ischemia/reperfusion were cytotoxic to human breast carcinoma BT549 cells similarly to colorectal cancer cells, and that galectin-3 protects BT549 cells against nitrogen free radical-induced cell death.
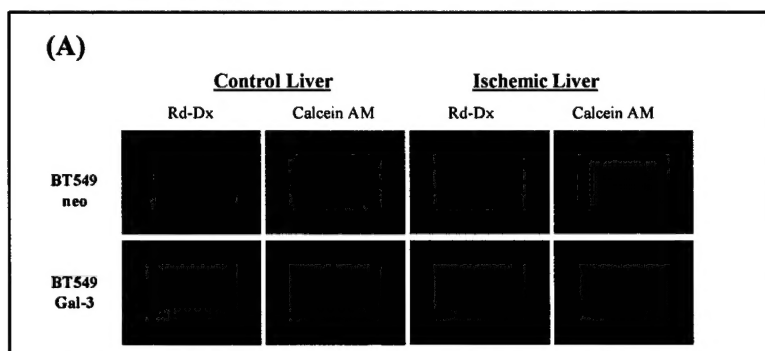

Fig. 1. Galectin-3 protects BT549 cell death induced by free radicals generated during hepatic ischemia/reperfusion.

(A) Rd-Dx and calcein AM-labeled BT549neo (top panels) and BT549Gal-3 (bottom panels) cells were co-cultured with the liver fragments isolated from control mouse liver (Control) or ischemic liver (Ischemic). Rd-Dx staining and calcein AM staining were detected with a maximum excitation/emission at 530/590 nm, and at 485/530 nm, respectively.

(B) After co-cultures of BT549neo or BT549Gal-3 cells with liver fragments, the numbers of cells containing Rd-Dx and calcein AM fluorescence were counted. The percentage of cell viability was determined by # cells containing both Rd-Dx and calcein AM fluorescence/ # cells containing Rd-Dx fluorescence. For NMMA treatment, mice received injections with 20 mg of NMMA via tail vein 30 min before liver harvest. Co-cultures were incubated in the presence of 1 mM NMMA. The assay was performed in triplicate and the results are presented as the mean ± SE.

(B)

| Cell Line	Liver Fragments	Treatment	Viability (%)
BT549 neo	Control		97.2 ± 7.9
BT549 neo	Ischemia		61.6 ± 9.4
BT549 neo	Ischemia	NMMA	92.2 ± 6.1
BT549 Gal-3	Control		96.9 ± 9.8
BT549 Gal-3	Ischemia		100

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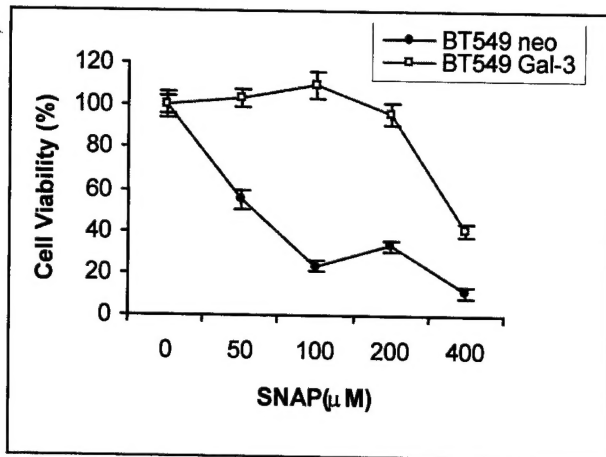
Figure 2. Galectin-3 inhibits SNAP-induced cell death in human breast epithelial cells. BT549neo and BT549Gal-3 cell cultures in the presence of 0, 50, 100, 200, or 400 μM SNAP for 48 hr. The number of live cells was determined by trypan blue exclusion assay. The percentage of cell viability was normalized to the respective untreated cells. All experiments were performed in triplicate and the error bars represent the standard deviation.

To further study galectin-3 inhibition of cell death induced by reactive nitrogen free radicals, we examined the role of galectin-3 in S-nitroso-N-acetylpeni-cillamine (SNAP)-induced cell death. SNAP is a NO donor known to induce cell death in many cell types including skeletal myoblasts, cortical neuronal cells, hepatocytes, and smooth muscle cells {reviewed in (25-27)}. Substantial cytotoxicity was induced by SNAP in BT549neo cells (Fig.2). After 48 hr treatment with 100 μM SNAP, approximately 80% of BT549neo cells died, whereas no significant cytotoxicity was detected in BT549Gal-3 cells following up-to 200 μM SNAP treatment for 48 hr. This demonstrated that galectin-expression protects human breast epithelial cells from nitric oxide-induced cell death. Next we investigated whether galectin-3 regulates the nitric oxide-induced apoptotic pathway. It is now well established that mitochondria and caspases play central roles in apoptosis regulation {reviewed in (28, 29)}. Mitochondrial events critical for apoptosis include disruption of electron transport, loss of mitochondrial transmembrane potential (Ψ_m), and release of cytochrome c (28, 29). To examine whether galectin-3 protects mitochondria integrity, we stained BT549neo and BT549Gal-3 cells with MitoTracker Red which selectively stains mitochondria and serves as a marker for the mitochondrial membrane potential (23). Twenty four hr treatment with 200 μM SNAP resulted in loss of mitochondria structure in ~90% of BT549neo cells. In contrast, more than 95% of the mitochondria in SNAP-treated BT549Gal-3 cells retained the fibrillar fluorescence pattern observed in the untreated cells, suggesting that galectin-3 overexpression protects cells against the loss of $\Delta\Psi_m$ (Fig. 3A). As predicted from the loss of mitochondrial integrity (Fig 3A), immunoblot analysis of cytosolic cytochrome c showed that the level of cytochrome release from the mitochondria was elevated in BT549neo cells as compared with BT549Gal-3 cells following 200 μM SNAP treatment (Fig. 3B).
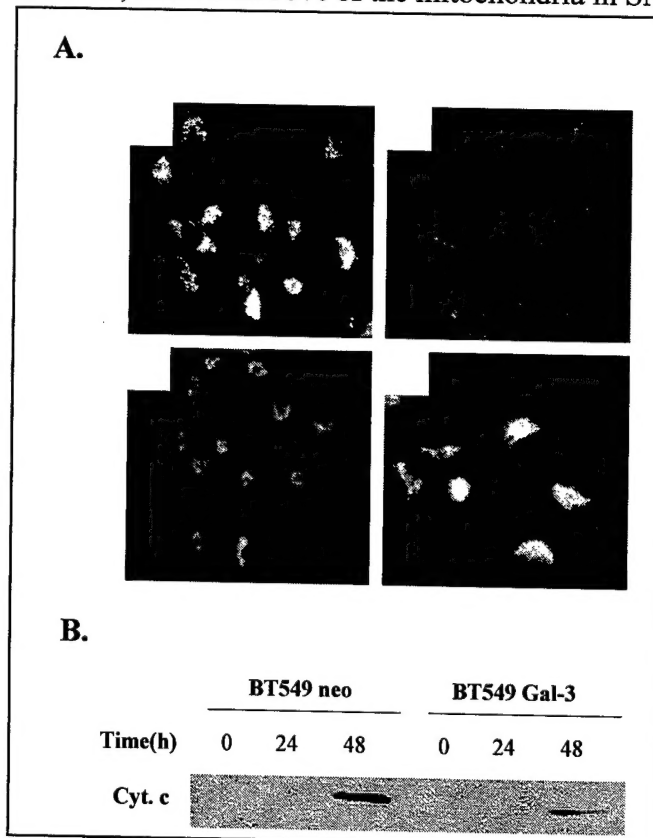
Fig 3. Galectin-3 protects mitochondria integrity and prevents cytochrome c release.
(A) BT549neo (a, b) and BT549Gal-3 (c, d) cells were plated on coverslips in six-well plates. Cells were treated with 0 (a, c) or 200 μM SNAP(b, d) for 24 hours, and stained with a fluorescent probe, MitoTracker Red. The mitochondria staining was examined with a Nikon Labophot microscope fitted with a digital video camera.
(B) Immunoblot analysis of cytosolic cytochrome c in BT549neo and BT549Gal-3 cells following 200 μM SNAP treatment for 0, 24, or 48 hr.

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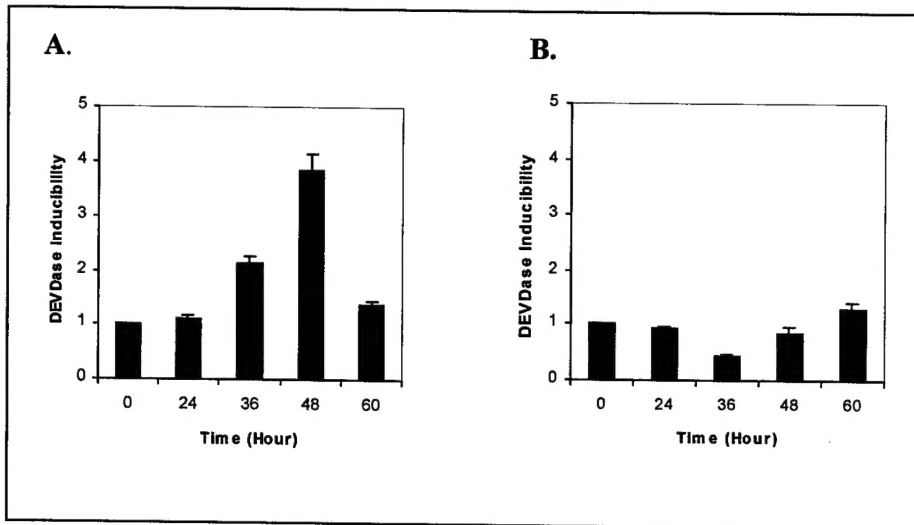
Fig. 4. Galectin-3 inhibits DEVDaseactivity
BT549neo (**A**) and BT549Gal-3 (**B**) cells grown in 60 mm dishes were treated with 200 µM SNAP. At indicated time points, the cells were washed with PBS and lysed with 200 µl caspase buffer as described in Materials and Methods. DEVDase activity in cytosol was determined by amc release from the tetrapeptide substrate Ac DEVD-amc, and DEVDase activity was normalized per µg protein. The DEVDase inducibility was normalized to the respective untreated cells. Three independent experiments were performed and the error bars represent standard deviation of the mean of triplicates.

Cytosolic cytochrome c translocated from the mitochondria was shown to be critical for caspase activation, a group of cysteine proteases that initiate the apoptotic process (30, 31). To further examine the effect of galectin-3 on nitric oxide-induced apoptosis, we measured caspase activity using the flurogenic substrate Ac-DEVD-amc, a substrate for caspase-3, -6, -7, -8, and –10. Caspase (DEVDase) activity was determined by amc release from the tetrapeptide substrate Ac-DEVD-amc. While SNAP treatment induced DEVDase activity ~4 fold in BT549neo cells following SNAP treatment, there was no induction in BT549Gal-3 cells following the same treatment (Fig. 4). These results demonstrate that galectin-3 inhibition of apoptotic cell death involves protection of mitochondria integrity, prevention of cytochrome c release, and inhibition of caspase activation.

(6) Key Research Accomplishments

During the first year (1999-2000) of the funding period, we demonstrated anti-apoptotic activity of galectin-3 in human breast epithelial cells. The present report showed that galectin-3 may rescue human breast epithelial cells from nitrogen free radical-induced cell death during liver ischemia/reperfusion. Previously , we also demonstrated that galectin-3 inhibits apoptosis induced by loss of cell adhesion (16).

(7) Reportable Outcomes

Publications:
1.   Lin, H-M., Moon, B-K., Yu, F. and **Kim, H.-R. C.** Galectin-3 mediates genistein-induced G_2/M arrest and inhibits apoptosis. Carcinogenesis 21: 1941-1945, 2000

2.   Moon, B.-K., Lee, Y. J., Battle, P., Jessup, J. M., Raz, A. and **Kim, H.-R. C**. Galectin-3 inhibits nitric oxide induced cell death in human breast carcinoma cells: Implication of galectin-3 function during metastasis. Am. J. Pathology, In press

(8) Conclusion

Taken together, we propose that galectin-3 is a critical determinant for anchorage-independent and free radical-resistant cell survival of disseminating cancer cells in the circulation during metastasis. This might explain why enhanced galectin-3 expression is often associated with the metastatic phenotype (1-6, 12, 13).

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Appendix
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Galectin-3 mediates genistein-induced G₂/M arrest and inhibits apoptosis

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Many recent studies have focused on potential chemopreventive activities of dietary genistein, a natural isoflavonoid compound found in soy products. Genistein has been implicated in anticancer activities, including differentiation, apoptosis, inhibition of cell growth and inhibition of angiogenesis. In previous studies, genistein was shown to induce apoptosis and cell cycle arrest at G₂/M in several cancer cell lines *in vitro*, which is associated with induction of p21[WAF1/CIP1], a universal inhibitor of cyclin-dependent kinases. At present, the molecular basis for diverse genistein-mediated cellular responses is largely unknown. In the present study, we investigated whether galectin-3, an anti-apoptotic gene product, regulates genistein-mediated cellular responses. We show that genistein effectively induces apoptosis without detectable cell cycle arrest in BT549, a human breast epithelial cell line which does not express galectin-3 at a detectable level. In galectin-3 transfected BT549 cells, genistein induced cell cycle arrest at the G₂/M phase without apoptosis induction. Interestingly, genistein induces p21[WAF1/CIP1] expression in galectin-3-expressing BT549 cells, but not in control BT549 cells undergoing apoptosis. Collectively, the results of the present study suggest that galectin-3, at least in part, is a critical determinant for genistein-mediated cell cycle arrest and apoptosis, and genistein induction of p21[WAF1/CIP1] is associated with cell cycle arrest, but not required for apoptosis induction.

Introduction

Epidemiological studies suggest that the soybean isoflavonoid genistein (4,5,7-trihydroxyisoflavone phytoestrogen) may decrease the incidence of certain types of cancer and reduce the age-adjusted death rates from breast cancer (1–3). Consistently, genistein has been shown to mediate cell differentiation (4), inhibit angiogenesis (5,6) and be cytotoxic in a wide variety of cancer cell lines *in vitro* (4,7–10). Experiments in mice also provide evidence for genistein-mediated cytotoxic/cytostatic activity (11). Several possible mechanisms for genistein's anticancer activity have been suggested. These include inhibi-

Abbreviations: Ac-DEVD-amc, acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin; ER, estrogen receptor; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

tion of protein tyrosine kinase and topoisomerase (7,8,12,13). In contrast to the anticancer activity of genistein, genistein enhances carcinogen-induced tumor formation in the mouse colon (14). Similarly, maternal exposure to genistein dose-dependently increases carcinogen-induced mammary tumor progression in female rat offspring, mimicking the effects of estrogen exposure (15). These studies suggest that genistein may enhance tumorigenic potential in an organ-specific and/ or developmentally regulated manner. At present, the molecular basis for genistein-mediated diverse cellular effects is not known.

Genistein induces apoptosis and cell cycle arrest at G₂/M in a variety of cancer cell lines *in vitro*, which is associated with p21[WAF1/CIP1] induction, a universal inhibitor of cyclin-dependent kinases (4,10,16). Efforts have been made to determine the gene products critical for mediating genistein-induced cellular responses. Since early studies showed that genistein binds estrogen receptors (ERs) (32,33), the role of ERs on genistein-mediated effects in cancer cells was examined. No relationship was found between ER expression and genistein-induced cell cycle arrest or apoptosis (7). Similarly, inhibition of cell growth by genistein was shown to be independent of the tumor suppressor gene product p53 (16).

In the present study, we investigated whether galectin-3, an anti-apoptotic gene product, modulates genistein-mediated cellular responses. Galectins are a family of proteins that bind to galactose-containing ligands (17). Galectin-3, a 30 kDa member of the galectin family, is widely found in epithelial and immune cells, and is highly expressed in various human tumor cells, including breast cancer (18–22). Although the exact mechanism is unknown, galectin-3 expression is associated with neoplastic progression and metastatic potential (18–25). A recent study showed that galectin-3 inhibits anti-Fas antibody and staurosporine-induced apoptosis in T-lymphocytes (26). In accordance with this, we have demonstrated that galectin-3 inhibits apoptosis induced by cisplatin (27) and loss of cell adhesion (anoikis) (28), suggesting that the oncogenic activity of galectin-3 may involve apoptosis inhibition. Here, we report that genistein arrests cells at the G₂/M phase in galectin-3-expressing human breast epithelial cells without detectable apoptosis induction, while it rapidly induces apoptosis in the control cells without detectable cell cycle arrest. In addition, we provide evidence indicating that genistein-induced p21[WAF1/CIP1] is associated with cell cycle arrest at G₂/M but is not required for apoptosis.

Materials and methods

Cell culture and genistein treatment

The human breast cancer cell line BT549 was obtained from Dr E.W.Thompson, Vincent T.Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, DC. Galectin-3-transfected BT549 cells (BT549-Galwt) were previously established by introducing an expression vector containing human galectin 3 cDNA into BT549 parental cells (27,28). BT549 cells transfected with the control vector conferring neomycin resistance are referred to as BT549neo. BT549 cells expressing a mutant galectin-3 in which Gly[182]

of the NWGR motif was replaced with Ala (27,28) are referred to as BT549-Galm. Cells were cultured using DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 0.5 µg/ml fungizone in 95% air/5% CO_2 at 37°C. After cells had been seeded for 4–6 h, cells were treated with 45 or 90 µM genistein (Sigma, St Louis, MO).

Antibodies

An anti-human galectin-3 monoclonal antibody (mAb) was purchased from the American Type Culture Collection (Rockville, MD), anti-cyclin D_1 mAb (Ab2) and anti-p21$^{WAF1/CIP1}$ mAb from Oncogene Research (Cambridge, MA), anti-p27^{KIP1} polyclonal antibody from Santa Cruz Biotech (Santa Cruz, CA), and anti-human β-actin mAb from Sigma.

Immunoblot analysis

Cell lysates were prepared using sodium dodecyl sulfate (SDS) lysis buffer (2% SDS, 125 mM Tris–HCl pH 6.8, 20% glycerol). The lysates were boiled for 5 min and then clarified by a 20 min centrifugation at 4°C. Protein concentration was measured using BCA protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein samples in SDS sample buffer (1% SDS, 62.5 mM Tris–HCl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 0.05% Bromophenol Blue) were boiled for 5 min and subjected to reducing SDS–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The blot was blocked with 5% non-fat dry milk in 100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.02% NaN_3 and 0.2% Tween-20 (T-TBS) for 1 h at room temperature. The membranes were incubated with the appropriate primary antibody in 5% milk in T-TBS. After three washes with T-TBS, the blot was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The antigen was detected using the enhanced chemiluminescence detection system (Pierce) according to the manufacturer's instruction.

Determination of cell cycle distribution

Cells were trypsinized, washed with PBS and fixed with 70% ethanol. The fixed cells were spun down and resuspended in Hoechst staining solution at a concentration of 1×10^6 cells/ml and incubated for 3 min at room temperature. The Hoechst staining solution consisted of 3 mg/ml Hoechst 33258 (Sigma) in Tris buffer (2 mM $MgCl_2$, 0.1% Triton X-100, 154 mM NaCl, 100 mM Tris, pH 7.5). The percentage of cells in each cell cycle phase was determined at the Imaging Flow Cytometry Core Facility at our institute.

Nuclear staining

Cells grown on coverslips were treated with 90 µM genistein. After 48 h of treatment, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for ≥12 h at 4°C. The coverslips were removed from the tissue culture dish and cells were exposed to 1 µg/ml 2'-(4-hydroxyphenyl)-5-(4-methyl-1 piperazinyl)-2,5'-bi-^{1}H-benzimidazole trihydrochloride pentahydrate (bisbenzimide, Hoechst 33258; Molecular Probes, Eugene, OR) in PBS for 15 min at room temperature, and washed with PBS. The cells were mounted in 0.1% phenylene diamine and 90% glycerol in PBS. Nuclear morphology was examined under UV illumination on a fluorescence microscope.

DEVDase activity assay

Cells were lysed in 50 mM Tris buffer (pH 7.5) containing 0.03% Nonidet and 1 mM dithiothreitol (DTT). Nuclei were removed by low-speed centrifugation (800 × g, 5 min), and the cytosol fraction was incubated with 40 µM acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-amc), 10 mM HEPES (pH 7.5), 50 mM NaCl and 2.5 mM DTT in a total volume of 200 µl for 60 min at 37°C. Fluoromethylcoumarin fluorescence, released by DEVDase (caspase) activity, was measured using 360 nm excitation. A CCD device (Instaspec IV; Oriel, Stratford, CT) fitted with a monochromator was used to measure the fluorescence emission spectrum. The intensity at the optimum wavelength (~460 nm) was measured. DEVDase activity was normalized per microgram of protein determined using a BCA protein assay kit (Pierce).

Results

Galectin-3 expression protects human breast epithelial cells against genistein-induced cytotoxicity

To examine the roles of galectin-3 on genistein-induced cyto-toxic/cytostatic activity, control and galectin-3-overexpressing BT549 cells (BT549neo and BT549-Galwt, respectively) were treated with genistein and subsequently evaluated for their cell viability using the MTS cell proliferation assay (Promega,
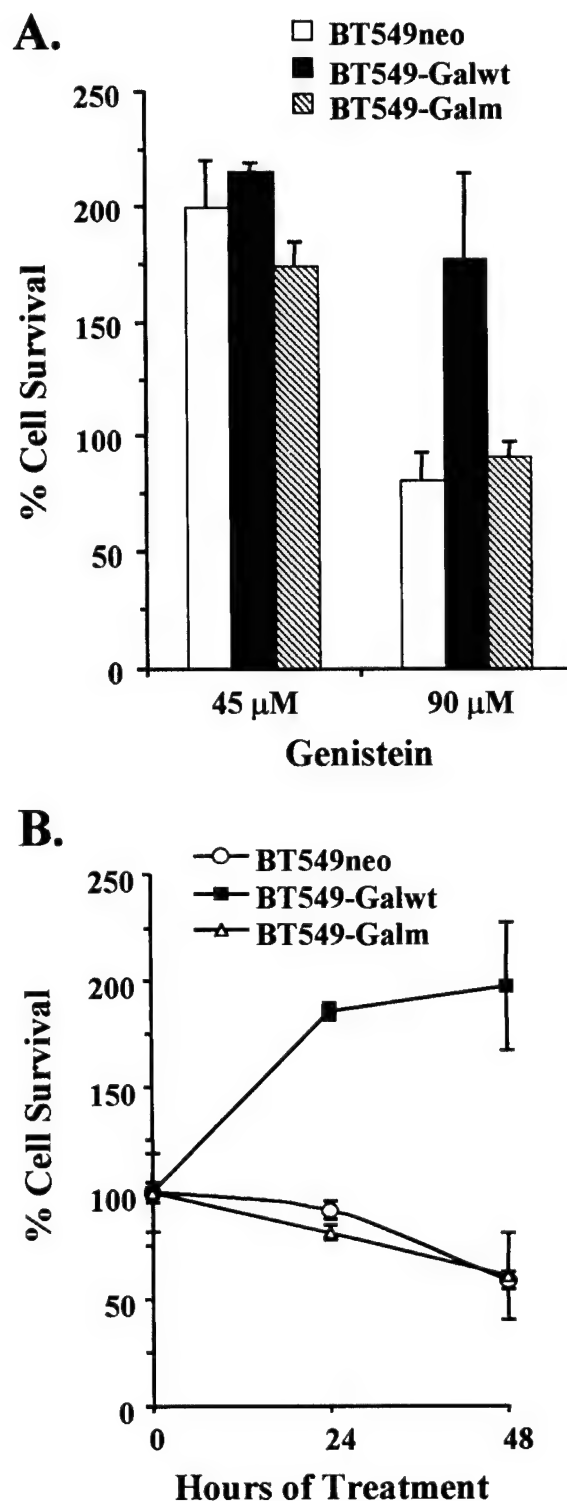
Fig. 1. Galectin-3 protects BT549 cells from genistein-induced cytotoxicity. BT549neo, BT549-Galwt and BT549-Galm cells were treated with 45 or 90 µM genistein for 24 h (**A**) or with 90 µM genistein for 24 or 48 h (**B**). The percentage of cell survival was normalized to the respective control cells (no treatment). All experiments were performed in triplicate; the error bars represent the standard deviation.

Madison, WI). These cells were chosen to study the roles of galectin-3 on genistein-mediated cellular effects, since the parental BT549 cells do not express galectin-3 at a detectable level. After exposure to 90 µM genistein for 24 h, ~80% of BT549neo cells remained viable, while the number of BT549-

A.

B.
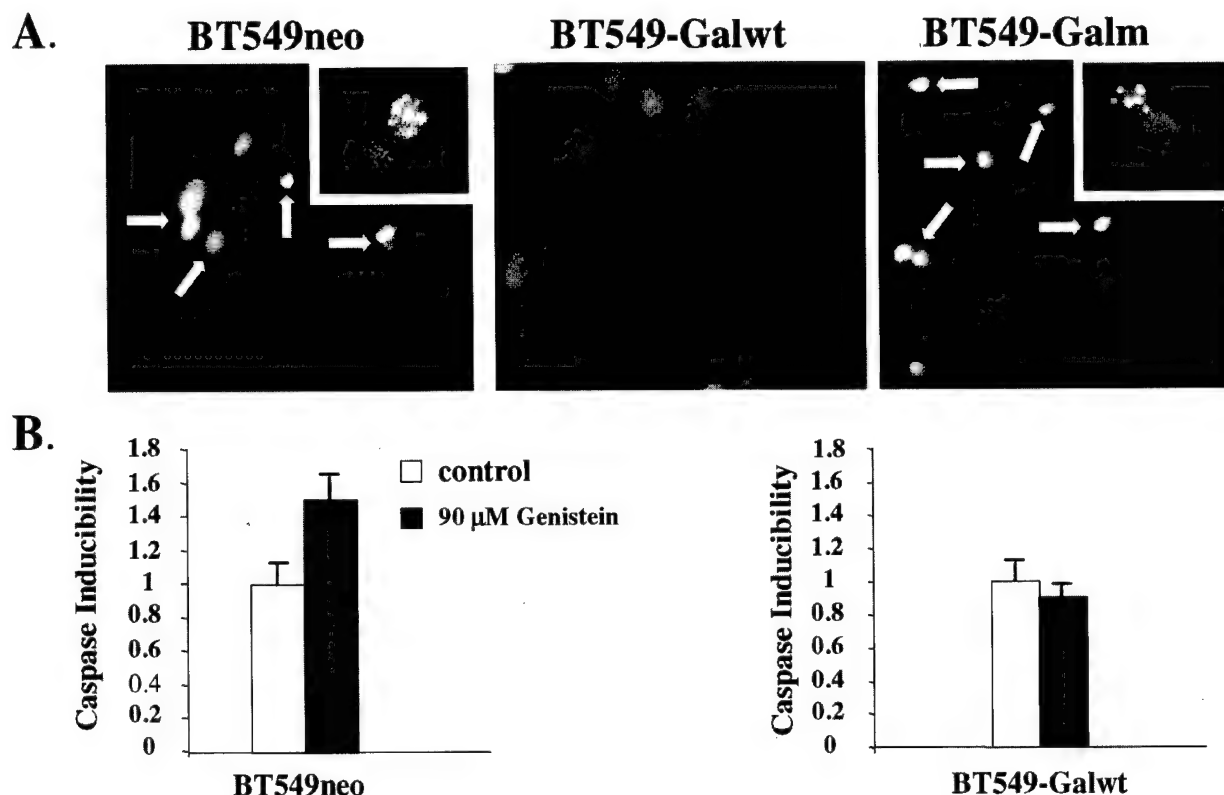
Fig. 2. Galectin-3 inhibits genistein-induced apoptosis. (A) BT549neo, BT549-Galwt and BT549-Galm cells were treated with 90 µM genistein for 48 h and analyzed for nuclear morphology using bisbenzimide staining. Arrows, apoptotic nuclei. Higher magnifications of the fragmented apoptotic nuclei of BT549neo and BT549-Galm cells are shown in the top, right corner. (B) BT549neo and BT549-Galwt cells were treated with 90 µM genistein for 48 h and DEVDase activity was assayed using Ac-DEVD-amc as a substrate. DEVDase activity was normalized per µg protein. The error bars represent standard deviation of the mean of triplicates.

Galwt cells increased to ~180% (Figure 1A). An additional 24 h exposure of cells to 90 µM genistein was cytostatic towards BT549-Galwt cells and was cytotoxic towards BT549neo cells (Figure 1B). This shows that galectin-3 expression protects BT549 cells against genistein-induced cytotoxicity.

Structurally, galectin-3 is composed of two distinct domains: an N-terminal domain containing proline- and glycine-rich sequences and a globular C-terminal domain containing the carbohydrate recognition site (18). Galectin-3 contains four amino acid residues, NWGR, that are conserved in the BH1 domain of the Bcl-2 family. This motif is critical for Bcl-2's anti-apoptotic activity (29). As in the Bcl-2 protein, substitution of the Gly^{182} residue with Ala in the NWGR motif of galectin-3 abrogates its anti-apoptotic function (27,28). To test whether the NWGR motif is required for galectin-3 inhibition of genistein-mediated cytotoxicity, we examined the effect of genistein on BT549-Galm cells, BT549 cells expressing mutant galectin-3 in which the Gly^{182} of the NWGR motif was substituted with Ala (27,28). As shown in Figure 1, mutant galectin-3 failed to protect BT549 cells against genistein-induced cytotoxicity, further substantiating the observation that the NWGR motif is critical for galectin-3's ability to prevent cell death.

Galectin-3 inhibits genistein-induced apoptosis and results in cell cycle arrest at G$_2$/M

To determine the mode of genistein-induced cell death, we examined nuclear morphology following genistein treatment. Genistein-treated BT549neo and BT549Galm cells underwent apoptotic changes, including chromosome condensation and fragmentation. In contrast, no significant change was observed in BT549-Galwt cells following genistein treatment (Figure 2A), suggesting that galectin-3 inhibits genistein-induced apoptosis. To confirm this, we measured caspase activity using the fluorogenic substrate Ac-DEVD-amc, a substrate for caspases 3, 6, 7, 8 and 10. Caspases are a family of cysteine proteases and their activation is regarded as the molecular instigator of apoptosis (30). Caspase activity in BT549neo and BT549-Galwt cells was determined by release of aminomethyl coumarin from the tetrapeptide substrate Ac-DEVD-amc. While genistein significantly induced DEVDase activity in BT549neo, there was no induction in BT549-Galwt cells. These results show that galectin-3 inhibits genistein-induced caspase activity and apoptosis in BT549 cells.

We have previously showed that galectin-3 inhibition of apoptosis is associated with its ability to arrest the cell cycle: cisplatin treatment or loss of cell anchorage induces cell cycle arrest at late G$_1$ in galectin-3-overexpressing cells, while it rapidly induces apoptosis in control cells (27,28). Next, we asked if the lack of apoptosis in BT549-Galwt cells following genistein treatment is also associated with galectin-3 involvement in cell cycle regulation. Genistein treatment of BT549neo cells resulted in a rapid decrease in the G$_0$/G$_1$ population (Figure 3A), and an increase in S phase and sub-G$_1$ (apoptotic) populations, suggesting that genistein triggers both cell cycle entry and apoptosis in these cells. However, genistein treatment reduced the G$_2$/M population of BT549neo cells. This suggests that these cells underwent apoptotic cell death before they reach the G$_2$/M phase. Genistein treatment of BT549-Galwt

cells also induced cell cycle entry, as detected by a reduction in G_0/G_1 population (Figure 3B). In contrast to BT549neo cells, no increase in the sub-G_1 population was detected in genistein-treated BT549-Galwt cells. Instead, with increased exposure time to genistein, BT549-Galwt cells accumulated at G_2/M, with reduced G_0/G_1 and S populations. These studies demonstrate that galectin-3 is a critical determinant for genistein-induced apoptosis and cell cycle arrest at G_2/M in BT549 cells.

$p21^{WAF1/CIP1}$ induction is associated with genistein-induced G_2/M arrest, but not with apoptosis

We previously reported that galectin-3 inhibition of anoikis involves cell cycle arrest at late G_1 through induction of cyclin D_1 (an early G_1 cyclin) and cyclin-dependent kinase inhibitors ($p21^{WAF1/CIP1}$ and $p27^{KIP1}$) (28). To understand the molecular basis for genistein-mediated cell cycle arrest at G_2/M in BT549-Galwt cells, the effect of galectin-3 expression on gene expression of cell cycle regulators was examined. Galectin-3 expression resulted in a basal level increase in cyclin D_1, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ as shown in Figure 4A. Genistein treatment further enhanced expression of cyclin D_1 in BT549-Galwt cells (Figure 4B), which is consistent with increased

cell cycle entry following genistein treatment. It should be noted that expression of INK-family inhibitors ($p15^{INK4B}$, $p16^{INK4A}$ and $p19^{INK4D}$), known to inhibit cyclin D_1 activity, was neither detected nor altered in BT549-Galwt cells (data not shown). While $p21^{WAF1/CIP1}$ is a universal inhibitor of cyclin-dependent kinases and causes cell cycle arrest at G_1/S or at G_2/M, $p27^{KIP1}$ is mostly involved at the G_1/S checkpoint, but not G_2/M (31). Although galectin-3 increased the basal level of $p27^{KIP1}$, $p27^{KIP1}$ expression is drastically down-regulated in BT549-Galwt cells following genistein treatment (Figure 4C), in agreement with rapid cell cycle progression beyond the G_1/S checkpoint. Genistein treatment of BT549-Galwt cells further induced $p21^{WAF1/CIP1}$ expression as shown in Figure 4B. Collectively, these results suggest that galectin-3 induction of $p21^{WAF1/CIP1}$ is in part responsible for genistein-mediated cell cycle arrest at G_2/M in BT549 cells. It should be noted that genistein had no effect on $p21^{WAF1/CIP1}$ expression in BT549neo and BT549-Galm cells, both apoptosis-prone cells. This clearly suggests that $p21^{WAF1/CIP1}$ expression is not required for genistein-induced apoptosis.

Discussion

Increasing evidence suggests that apoptosis regulation is tightly linked to cell cycle regulation. Although apoptosis can be induced at any point during the cell cycle, apoptosis sensitivity varies greatly at different points in the cell cycle. Genistein has previously been shown to induce cell cycle arrest at G_2/M and apoptosis in many cancer cell lines (4,10,16). It has been suggested that $p21^{WAF1/CIP1}$ is critical for both genistein-induced cell cycle arrest and apoptosis. The present study, however, indicates that genistein-induced $p21^{WAF1/CIP1}$ may be associated with apoptosis inhibition through cell cycle arrest rather than apoptosis induction. Our study clearly suggests that the level of expression of anti-apoptotic gene products such as galectin-3 is a critical determinant of genistein-induced cell cycle arrest or apoptosis. This is of particular importance in the light of the recently reported potential carcinogenic activity of genistein (14,15). Genistein-induced apoptosis seems to be accompanied by induction of cell cycle entry (Figure 3). When genistein-induced apoptosis is inhibited by
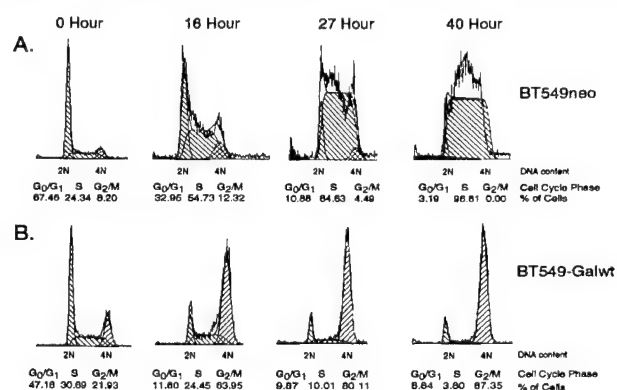
Fig. 3. Galectin-3 mediates cell cycle arrest at G_2/M arrest. Flow cytometric cell cycle histograms of BT549neo (**A**) and BT549-Galwt (**B**) treated with 90 µM genistein for 0, 16, 27 and 40 h. The proportions of cells in each cell cycle phase are presented.
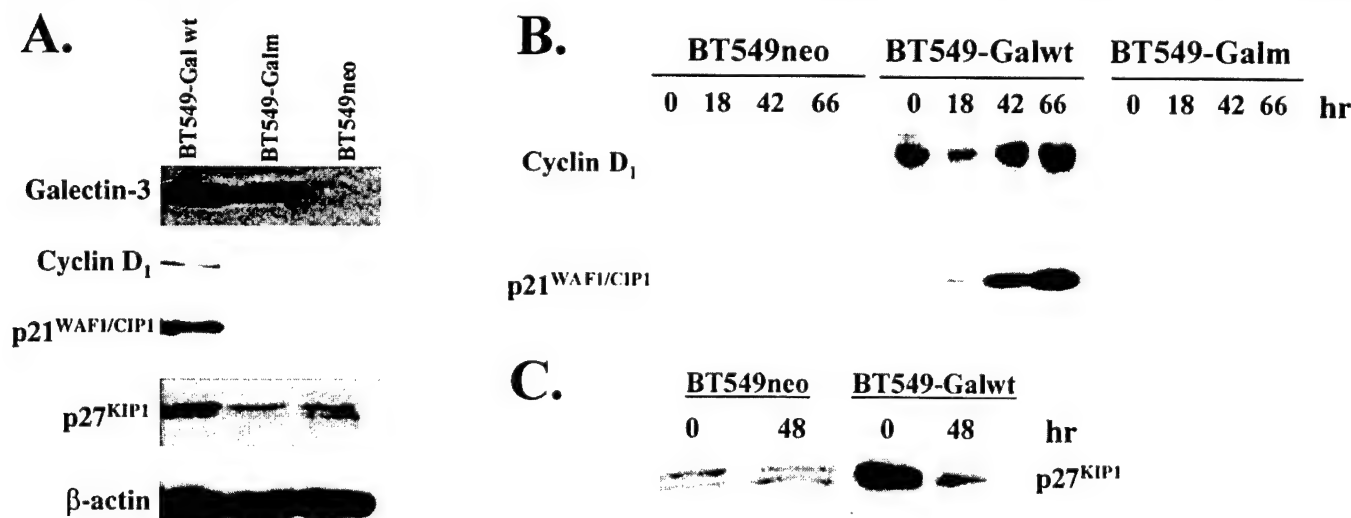
Fig. 4. Galectin-3 induction of $p21^{WAF1/CIP1}$ is associated with G_2/M arrest. Immunoblot analysis of galectin-3, cyclin D_1, $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and β-actin in BT549neo, BT549-Galwt or BT549-Galm cells. Protein samples were prepared from cells without treatment (**A**) or with 90 µM genistein treatment for the indicated times (**B** and **C**).

galectin-3, galectin-3 induction of cyclin D_1 and rapid cell cycle progression beyond the S phase may enhance accumulation of genetic mutations contributing to carcinogenesis.

Previous studies and the present one suggest that galectin-3 results in cell cycle arrest at different points depending on the apoptotic stimuli. Galectin-3 induces cell cycle arrest at late G_1 in response to cisplatin treatment or loss of cell adhesion (anoikis) (27,28), whereas it induces G_2/M arrest following genistein treatment. At present it is not known how galectin-3 modulates the expression of cell cycle regulatory genes, including cyclin D_1, p21$^{WAF1/CIP1}$ and p27^{KIP1}. Galectin-3 is expressed in the nucleus and cytoplasm and also in secreted form. Galectin-3 in the nucleus may be involved in the regulation of gene expression. It is equally possible that cytoplasmic galectin-3 or extracellular galectin-3 induces signal transduction leading to modulation of gene expression. Cisplatin treatment or anoikis further enhanced p21$^{WAF1/CIP1}$ and p27^{KIP1} expression in galectin-3-expressing cells, resulting in G_1/S arrest. In contrast, genistein enhanced p21$^{WAF1/CIP1}$ expression only, while it abolished galectin-3 induction of p27^{KIP1}, leading to G_2/M arrest. Specific interactions between apoptosis initiation signaling and galectin-3 regulation of gene expression remain to be fully investigated.

In summary, the present study provides a mechanistic insight into genistein-induced cell cycle arrest or apoptosis regulated by the anti-apoptotic gene product galectin-3. Taken in conjunction with the finding that galectin-3 is often overexpressed in human cancer (18–20,22–25), our finding may be critical for understanding the chemopreventive/chemotherapeutic potentials of genistein.

Acknowledgements

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Research for the period 1 Oct 00 - 1 Jul 01

(4) Introduction

Galectin-3 is a member of the β-galactoside-binding family which is found widely in epithelial and immune cells. The expression of galectin-3 is associated with neoplastic progression and metastatic potential (1-8), suggesting a role in oncogenesis . Galectin-3 modulates a variety of cellular processes. Extracellular galectin-3 mediates cell migration, cell adhesion and cell-cell interactions, while nuclear galectin-3 is involved in pre-mRNA splicing (9-12). Interestingly, recent studies showed that cytoplasmic, but not nuclear, galectin-3 is associated with tumor progression (13, 14). Yet, the function of cytoplasmic galectin-3 is unknown.

We, and others, have previously shown that galectin-3 inhibits T-cell apoptosis induced by anti-Fas antibody and epithelial cell apoptosis induced by staurosporine, cisplatin, genistein, and anoikis (15-20). The anti-apoptotic activity of galectin-3 was also demonstrated in galectin-3 deficient mice. Peritoneal macrophages from galectin-3 deficient mice were more sensitive to apoptotic stimuli than those from control mice (21).

Explosive progress towards dissecting the molecular basis for the regulation of the apoptosis commitment step has been made during the past decade. Two major apoptotic pathways, intrinsic and extrinsic pathways, have been defined. Intrinsic apoptotic signaling induces cytochrome c release from the mitochondria. Cytosolic cytochrome c initiates the formation of a ~700 kDa complex, called the "apoptosome", which consists of cytochrome c, caspase adaptor protein such as Apaf-1, and caspases (22, 23). Caspase-9, an upstream caspase, is first activated in the apoptosome by autoproteolytic cleavage, followed by subsequent activation of downstream effector caspases such as caspase-3 and –7. The effector caspases appear to be recruited and activated within the apoptosome, and then released to the ~200-300 kDa "micro-apoptosome" complex (24-26). The extrinsic apoptotic signals are mediated by cell surface death receptors including Fas, tumor necrosis factor (TNF) and TRAIL receptor families. The death domain in the death receptor initiates the formation of the "death inducing signaling complex (DISC)", where caspase-8, an upstream caspase, is first activated, followed by the subsequent activation of effector caspases (reviewed in (27)). Thus, caspase-9 and –8 activate the caspase cascade following intrinsic and extrinsic apoptotic signals respectively. In addition, recent studies showed that caspase-8 is required during the cytochrome c-mediated intrinsic apoptotic pathway and possibly, for apoptotic signal amplification (28).

Although a critical role for galectin-3 in apoptosis inhibition is now well documented, neither the functional site nor the molecular mechanism of how galectin-3 regulates apoptosis is understood. The ability of galectin-3 to protect epithelial cells against intrinsic apoptosis induced by agents working through different mechanisms suggests that galectin-3 regulates the apoptosis commitment step, a common pathway involving caspase and mitochondria. In the present study, we examined the subcellular location of galectin-3 during intrinsic apoptosis. We also investigated the molecular action of galectin-3 in the protection of mitochondrial integrity and the regulation of caspase activation in the apoptosome and the micro-apoptosome.

(5) Body of Report

Methods

Cell culture and reagents.

The human breast cancer cell line BT549 was obtained from Dr. E. W. Thompson, Vincent T. Lombardi Cancer Research Center, Georgetown University Medical Center (Washington, D. C.). Galectin-3 transfected BT549 cells (BT549Gal-3) were previously established by introducing an expression vector containing human galectin-3 cDNA into BT549 parental cells (16, 17). The neo-resistant control vector transfected BT549 cells are referred to as BT549neo. Cells were cultured using DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.5 μg/ml fungizone in a 95% air and 5% CO_2 incubator at 37^0C. All cell culture reagents were purchased from Invitrogen inc. (CA, USA)

DEVDase, IETDase, and LEHDase activity assay

Cells were lysed in CEB (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) containing 0.03% Nonidet. Lysates were centrifuged at 15,000 g for 10 min, and 50 μl cytosol fraction was incubated for 60 min at 37 ^{0}C in a total volume of 200 μl caspase buffer [10 mM

HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT] containing 25 µM acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC, Bachem, PA), acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin (Ac-IETD-AFC) or acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (Ac-LEHD-AFC). Using Spectra Maxi Germini fluorescence plate reader (Molecular Devices, CA), AMC or AFC fluorescence, released by caspase activity, was measured at 460 nm using 360 nm excitation or at 505 nm using 400 nm excitation wavelength, respectively. Caspase activity was normalized per microgram of protein determined by BCA protein assay kit (Pierce, IL).

Mitochondria staining

Cells were plated on a coverslip in a 12-well plate. After 24 hr of apoptosis induction, the cells were incubated with media containing 250 nM MitoTracker Red (Molecular Probes, Eugene, OR) for 30 minutes at 37^0C. Cells were washed with PBS, fixed with 3.7% paraformaldehyde in PBS for 15 min at 37^0C. The coverslips were mounted onto glass slides with anti-fade solution (Molecular Probes, OR). Fluorescent staining of the mitochondrial membrane was examined with a Nikon Labophot microscope fitted with a digital video camera (Photometrics, Tucson, AZ) or a Zeiss LSM 310 microscope (Carl Zeiss, Germany) in the confocal mode.

Cytochrome c release

Cells were harvested at 0, 24, and 48 hr following treatment with 24 µM cisplatin, resuspended in ice-cold CEB (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 4 mM dithiothreitol) containing 250 mM sucrose and Protease Inhibitor cocktail (Roche, Mannheim, Germany), and incubated for 1 hr at 4^0C. The lysates were then passed through a 26 1/2 gauge syringe 15 times and then centrifuged at 15,000 g for 20 min at 4^0C. The resulting supernatant was analyzed by immunoblot analysis using anti-cytochrome c antibody (clone 7H8.cC12, ZYMED Laboratories Inc, CA).

Confocal immunofluorescence microscopic analysis

Cells were cultured on coverslips to 75% confluency. Apoptosis was induced by treatment with 25 µM cisplatin for 24 hr or 0.5 µM staurosporine for 150 minutes, or by growth factor withdrawal for 48 hrs. Cells were washed with PBS three times, fixed with 3.7% formaldehyde in PBS for 15 minutes, washed with PBS-S (PBS-0.1% saponin), and then incubated with 1% BSA in PBS-S for 1 hr. After 6-washes with PBS-S, cells were incubated with rat anti-galectin-3 antibody (ATCC, VA) or anti-cytochrome c antibody (clone 6H2.B4, BD PharMingen, CA) for 2 hours at room temperature. After 6-washes with PBS-S, the cover slip was incubated with FITC conjugated secondary antibodies (Sigma, MO) for 1 hour. After 6-washes with PBS-S, the cover slip was mounted up-side-down with anti-fade solution (Molecular Probes, OR), sealed, and examined under a Zeiss LSM 310 microscope in the confocal mode.

Immunoblot analysis

Cell lysates were prepared using SDS lysis buffer (2% SDS, 125mM Tris-HCl, pH 6.8, 20% Glycerol). The lysates were boiled for 5 min and then clarified by a 20-minute centrifugation at 4°C. Protein concentration was measured using BCA protein assay reagent (Pierce, IL). Equal amounts of protein samples in SDS sample buffer (1 SDS, 62.5mM Tris-HCl, pH 6.8, 10% Glycerol, 5% β-mercaptoethanol, 0.05% Bromophenol Blue) were boiled for 5 minutes and subjected to reducing SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulos membrane. The blot was blocked with 5% nonfat dry milk in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% Na and 0.2% Tween-20 (T-TBS) for 1 hour at room temperature. The membranes were incubated with the appropriate primary antibody in 5% milk in T-TBS. After three washes with T-TBS, the blot was incubated with the appropriate HRP-conjugated secondary antibody. The antigen was detected using the ECL detection system (Pierce, Rockford, I according to the manufacturer's instruction.

Isolation of Mitochondria

Mitochondria were isolated as previously described (29). Briefly, BT549Gal-3 cells were homogenated in CEB with 250 mM sucrose to protect mitochondria by douncing 20 times with a type B dauncers (Knotes Glass Company, NJ). Homogenates were centrifuged at 750 g for 3 X 10 min at 4 ^{0}C to remove debris and nuclei. Then the supernatant was centrifuged at 15,000g for 20 min, the pellet, which contains mitochondria, was lysed in SDS lysis buffer and 20 µg mitochondrial proteins were subjected to immunoblot analysis.

Size-exclusion chromatography

The apoptosome and micro-apoptosome complexes were isolated by size-exclusion chromatography using a HiPrep 16/60 S-300 Sephacryl high-resolution column (Amersham Pharmacia Biotech) as previously described (24). The column was equilibrated with 5% (w/v) sucrose, 0.1% (w/v) CHAPS, 20 mM HEPES/NaOH, 5 mM dithiothreitol, pH 7.0, and calibrated with an Amersham Pharmacia Biotech HMWgel filtration protein standards kit containing thyroglobulin (MW 669,000), ferritin (MW 418,000), catalase (MW 206,000), and bovine serum albumin (MW 67,000) protein standards. One ml of 10 mg/ml of control and apoptotic cell lysates of BT549Gal-3 and BT549neo were applied onto the column, and eluted from the column at a flow rate of 0.5 ml/min. All separations were carried out at 4°C and 2 ml fractions were collected. Fifty µl of each fraction was assayed for DEVDase, IETDase and LEHDase activity.

Apoptosome formation by exogenous cytochrome c

BT549Gal-3 cells were lysed in CEB by passing through 26 ½ gauge needle for 15 times. The protein concentration of the lysate was measured using BCA protein assay kit and adjusted to 4 µg/µl. Cytochrome c was added into cell lysates to the final concentration of 50 µg/ml and incubated on ice for 30 minutes. Twenty µl of lysates without or with cytochrome c were mixed with 5 µl of 5X native protein loading buffer (25 % glycerol, 0.25% Bromophenol blue) and loaded onto a pre-chilled native gradient (4-12%) Tris-Glycine polyacrylamide gel and separated at 0°C using non-denaturing electrophoresis buffer (25 mM tris, 250 mM glycine pH 8.3). After electrophoresis, the gel was soaked in 0.5% SDS, 25 mM Tris, 250 mM glycine pH 8.3 for 10 min and blotted onto a nitrocellulose membrane. The nitrocellulose membrane was incubated with 0.1% SDS, 62.5 mM Tris pH 6.8, 1% β-mercaptoethanol at 70°C for 30 min. After washing with T-TBS for 3X for 10 min, the membrane was subjected to immunoblot analysis.

Results

Galectin-3 inhibits DEVDase (caspase-3 like) activity and protects mitochondrial integrity.

We previously showed that galectin-3 overexpression results in inhibition of poly(ADP-ribose) polymerase (PARP) cleavage following apoptotic stimuli (16, 17), suggesting that galectin-3 prevents caspase-mediated apoptosis. Since effector caspases (such as caspase-3, and –7) cleave PARP at the DEVD^{216}-G site, we measured cisplatin- and staurosporine-induced DEVDase activity in control (BT549neo) and galectin-3 overexpressing human breast epithelial cells (BT549Gal-3) using fluorogenic substrate Ac-DEVD-amc. DEVDase (caspase-3 like) activity increased ~5 fold at 48 hr following 25 µM cisplatin treatment in BT549neo cells, whereas it increased only ~ 2 fold following the same treatment in BT549Gal-3 (Fig. 1A). Similarly, DEVDase activity increased ~ 5 fold at 2.5 hr following 0.5 µM staurosporine treatment in BT549neo cells, whereas no significant increase was detected in BT549Gal-3 cells (Fig. 1B). These results showed that the caspase-3 (effector caspase)-like activity necessary for apoptosis execution is significantly inhibited by galectin-3 overexpression.

Mitochondrial events critical for apoptosis include the disruption of electron transport, loss of mitochondrial transmembrane potential (Ψ_m), and the release of cytochrome c (30), resulting in caspase activation. To examine whether galectin-3 protects mitochondria integrity, we stained BT549neo and BT549Gal-3 cells with MitoTracker Red which selectively stains mitochondria and serves as a marker for the mitochondrial membrane potential (31). Thirty six hr treatment with 25 µM cisplatin resulted in the loss of mitochondrial structure in BT549neo cells (Fig. 2A and B). In contrast, the mitochondria in cisplatin-treated

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BT549Gal-3 cells retained the fibrillar fluorescence pattern as observed in the untreated cells (Fig. 2C and D), suggesting that galectin-3 overexpression protects cells against the loss of $\Delta\Psi_m$. As predicted from the loss of mitochondrial integrity, the immunoblot analysis of cytosolic cytochrome c showed that the level of cytochrome c released from the mitochondria was elevated in BT549neo cells as compared with BT549Gal-3 cells following cisplatin treatment (Fig. 2E). To exclude the possibility that cytochrome c was released to the cytosolic fractions during the preparation of cellular homogenates involving passage through a 26 1/2 gauge syringe, we performed indirect immunostaining of cytochrome c in control and apoptotic cells. The majority of BT549neo cells exhibited diffused cytochrome c staining following the cisplatin treatment (Fig. 2G), growth factor withdrawal (Fig. 2H), and staurosporine (Fig. 2I). In contrast, cytochrome c staining in BT549Gal-3 cells remained punctuate following the same treatment (Fig. 2K,L,M). Confocal immunofluorescence microscopic analysis of cells co-stained with anti-cytochrome c Ab/ FITC conjugated secondary Ab and with MitoTracker Red confirmed that cytochrome c remained in the mitochondria in BT549Gal-3 cells following apoptotic stimuli, as shown by yellow staining (Fig. 2S,T,U). These results showed that galectin-3 overexpression protects cells from losing their mitochondrial membrane potential and prevents cytochrome c release to the cytosol.

Galectin-3 is redistributed onto the intracellular membranes following apoptotic stimuli.

To determine the subcellular location where galectin-3 exerts its anti-apoptotic effect, galectin-3 protein in the control and apoptotic cells was stained with anti-galectin-3 mAb and FITC conjugated secondary Ab. Galectin-3 staining was evenly detected in the nucleus and the cytoplasm in the control BT549Gal-3 cells (Fig. 3A). In contrast, galectin-3 staining displayed a compact and punctuated extranuclear membrane staining in cells treated with cisplatin (Fig. 3B), cultured in serum-free medium (Fig. 3C) or treated with staurosporine (Fig. 3D). This suggests that galectin-3, following apoptotic stimuli, translocates into the intracellular membrane, possibly mitochondria where it prevents mitochondrial dysfunction and inhibits caspase activation. To examine whether galectin-3 translocates to the mitochondria, cells were co-stained with anti-galectin-3 mAb and with MitoTracker Red. The galectin-3 staining patterns (Fig. 3B,C,D) strikingly resembled the mitochondrial staining patterns (Fig. 3 F,G,H) following apoptotic stimuli, but not in the control cells (Fig. 3A and E). Confocal microscopic analysis from the same plane of focus revealed that galectin-3 and mitochondria indeed co-localize following apoptotic stimuli as shown by yellow staining (Fig. 3J,K,L). To further confirm galectin-3 translocation to the mitochondria, mitochondria was isolated in the presence of 250 mM sucrose. Immunoblot analysis of galectin-3 using mitochondrial proteins confirmed a significant increase in the level of galectin-3 protein in the mitochondria following apoptotic stimuli (Fig. 3M).

Galectin-3 is found in the apoptosome and downregulates activation of caspase cascade.

The apoptosome has been defined as a ~700 kDa complex that catalyzes the activation of caspases (22-26). Formation of the apoptosome complex appears to occur at the perinuclear membrane (32, 33). Since galectin-3 translocates onto the perinuclear mitochondrial membrane (Fig. 3) and inhibits DEVDase activity (Fig. 1), we asked if galectin-3 physically associates with the apoptosome and regulates activation of caspase cascade. To this end, we performed size exclusion chromatographic analysis to isolate the apoptosome and micro-apoptosome from BT549neo and BT549Gal-3 cells in the absence or presence of apoptotic stimuli. The basal levels of DEVDase (caspase-3 like), IETDase (caspase-8 like) and LEHDase (caspase-9 like) activates (dotted lines in Fig. 3) were detected in fractions 39-42 from both BT549neo and BT549Gal-3 cells, which contains the apoptosome complex with a molecular weight of ~700 kDa. Following cisplatin treatment, most of the DEVDase activity was predominantly found in two large complexes, the ~700 kDa (apoptosome) and the ~200-300 kDa (micro-apoptosome) complexes (Fig.4A and D). The broad peak of DEVDase activity in the micro-apotosome from BT549neo cells was detected in fractions between 54 and 72, whereas the peak of DEVDase activity in BT549Gal-3 cells was tighter (fractions 56-65).

Next we examined the initiator caspase activity (caspase-8 and –9) in size fractionated apoptotic lysates. Increased IETDase (caspase-8 like) and LEHDase (caspase-9 like) activates from BT549neo apoptotic lysates were mainly in the apoptosome fractions (Fig. 4B and C). In cisplatin treated BT549Gal-3 lysates, IETDase

activity was drastically reduced in the apoptosome (Fig. 4E), while increased LEHDase activity was detected in the apoptosome from BT549Gal-3 (Fig. 4F). This suggests that downregulation of caspase-8 may be one of the major targets for galectin-3 in the inhibition of apoptosis. In addition, it should be noted IETDase and LEHDase activities in BT549Gal-3 apoptotic lysates were detected exclusively within the apoptosome (Fig. 4E and F), whereas a small amount of IETDase and LEHDase activity in BT549neo apoptotic lysates was detected in fractions 50-60 (Fig. 4B) and fractions 50-70 (Fig. 4C) respectively, which contain micro-apoptosome. This suggests that the release of IETDase and LEHDase activity from apoptosome to micro-apotosome is prevented in BT549Gal-3 cells.

To determine whether galectin-3 physically associates with the apoptosome and/or the micro-apoptosome, we performed an immunoblot analysis of galectin-3 in size fractionated lysates. Galectin-3 protein in BT549neo lysates was not detectable by immunoblot analysis (data not shown). In control BT549Gal-3 lysates, a 32 kDa galectin-3 monomer was barely detectable (Fig. 5). Galectin-3 was located in fractions corresponding to a Mr. of ~100-300 kDa, suggesting that it is complexed to other as yet undefined proteins or exists as an oligomer. Following apoptotic stimuli, galectin-3 was recruited/assembled into the ~700 kDa apoptosome complex (fractions 39-42). Galecti-3 was also found in micro-apotosome (fractions 50-70). Interestingly, the peak of DEVDase activity in the micro-apotosome from apoptotic BT549Gal-3 cells (Fig. 3D) was coincident with the decreased levels (or degradation) of galectin-3 in these fractions (fractions 60-63 in Fig 5).

The co-elution of galectin-3 with the apoptosome complex (Fig. 5) as well as the downregulation of IETDase activity (Fig. 4E) suggests a critical role for galectin-3 in caspase-8 regulation. To examine the role of galectin-3 on caspase-8 processing, we next performed an immunoblot analysis of caspase-8. In control BT549neo and BT549Gal-3 lysates, pro-caspase-8 with a Mr of 55 kDa was detected mostly in fractions 50-67 corresponding to a Mr of ~150-300 kDa, and a small amount of proform was present in the apoptosome (fractions 39-42 in Fig. 6A and C). Following apoptotic stimuli, the amount of the pro-caspase-8 protein significantly decreased and all of the pro-caspase-8 in the apoptosome from BT549neo lysates disappeared (Fig. 6B), which was accompanied by increased IETDase activity (Fig. 4B). In contrast, following apoptotic stimulation, the apoptosome fractions from BT549Gal-3 lysates still contained pro-caspase-8 (Fig. 6D) with no detectable IETDase activity (Fig. 3E). More interestingly, a partially processed intermediate form of caspase-8 with a Mr of ~27 kDa, which is enzymatically inactive, was accumulated in BT549Gal-3 cells (Fig. 6D). These results suggest that galectin-3 may donwregulate caspase-8 by interfering with its proteolytic processing.

To exclude the possibility that galectin-3 was coincidentally eluted in ~700 kDa fractions, we tested whether galectin-3 can be recruited into a cytochrome c-mediated apoptosome complex. To this end, we established a cell-free caspase activation system from BT549Gal-3 cells as previously described (34). DEVDase activity was increased in cell free extracts by adding 50 µg/ml exogenous cytochrome c followed by incubation at 37^0C. DEVDase activity was drastically induced by exogenous cytochrome c (Fig. 7A), confirming an establishment of a cell-free caspase activation system. To determine whether galectin-3 is recruited to the cytochrome c-containing apoptosome complex, cell extracts were incubated without or with 50 µg/ml cytochrome c. Incubation was performed at 0^OC to prevent caspase proteolytic processing which may result in disassociation of apoptosome component(s). The protein complex was analyzed by a native gradient (4-12%) Tris-Glycine polyacrylamide gel (Invitrogen, CA) electrophoresis and immunoblot analysis. As described in the Materials and Methods, the protein gel was denatured after electrophoresis, since both anti-galectin-3 and anti-cytochrome c antibodies preferentially detect denatured antigens. Galectin-3 was detected in a ~150 kDa protein complex in BT549Gal-3 lysates (Fig 7B, left panel), which is consistent with the result obtained by size exclusion chromatography (Fig. 3). Upon incubation with cytochrome c, the intensity of the ~150 kDa galectin-3 band decreased, which was accompanied by appearance of galectin-3 in a large complex corresponding to a Mr of ~700 kDa. Immunoblot analysis of cytochrome c revealed that this large molecular weight protein complex also contains cytochrome c (Fig. 7B, right panel), further confirming the galectin-3 involvement in the apoptosome complex.

(6) Key Research Accomplishments

Specific aims proposed in the DOD idea grant application are
1. To investigate the role of subcellular localization on the ability of galectin-3 to protect against apoptosis.
2. To investigate the effects of galectin-3 on the signal transduction pathways during anoikis.

During the funding period of 2000-2001, we investigated the functional site of galectin-3 for the regulation of apoptosis. We demonstrated that galectin-3 translocates onto the perinuclear membranes including mitochondria, protects mitochondrial integrity, and downregulates caspase activation in the apoptosome complex.

(7) Reportable Outcomes

Publications:
1. Yu, F., Raz, A and **Kim, H.-R. C.** Galectin-3 inhibits cytochrome c release and downregulates caspase activation. Submitted.

(8) Conclusion

Galectin-3 is a critical regulator of the apoptosis commitment step involving mitochondria and caspase cascade.

(9) References

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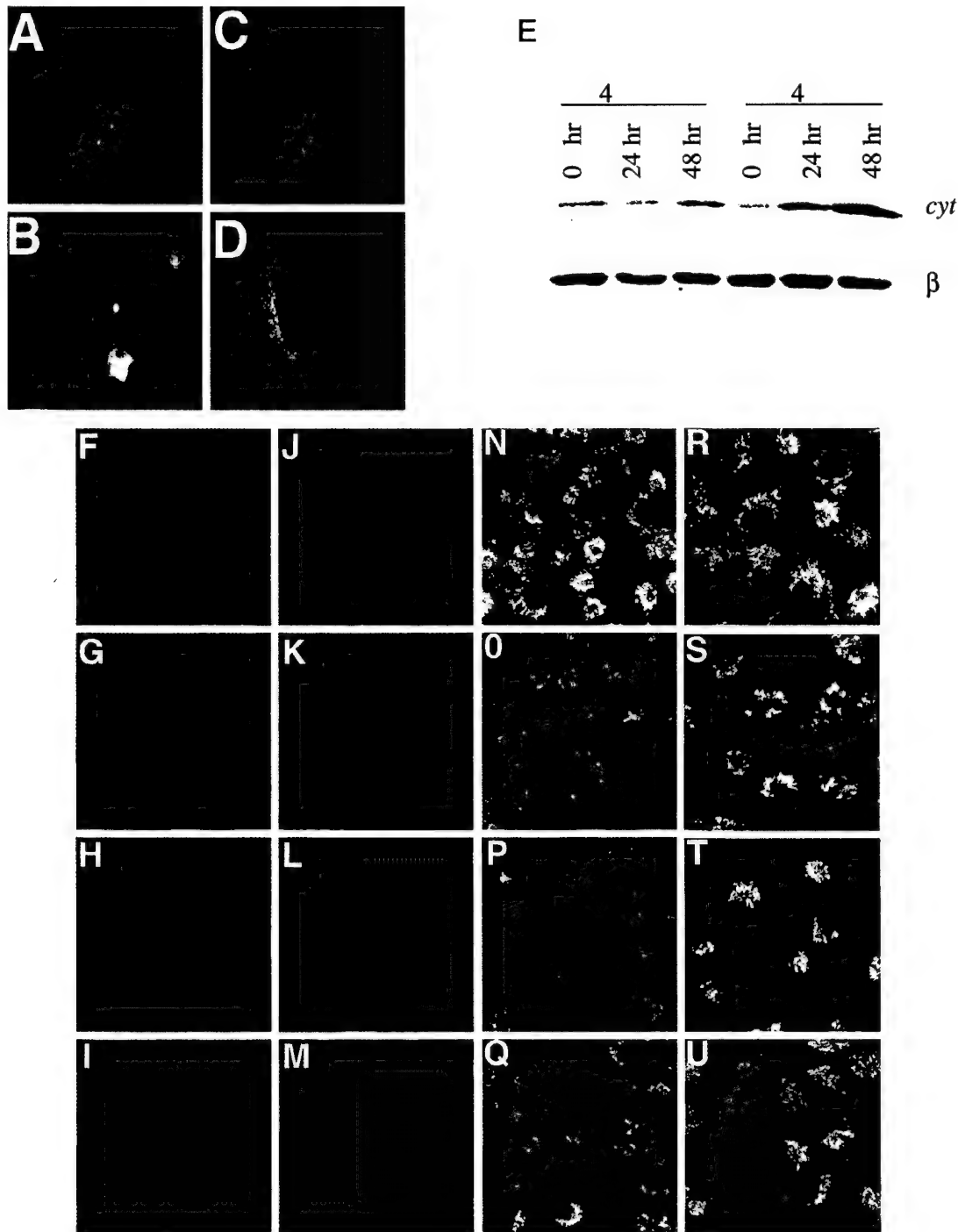
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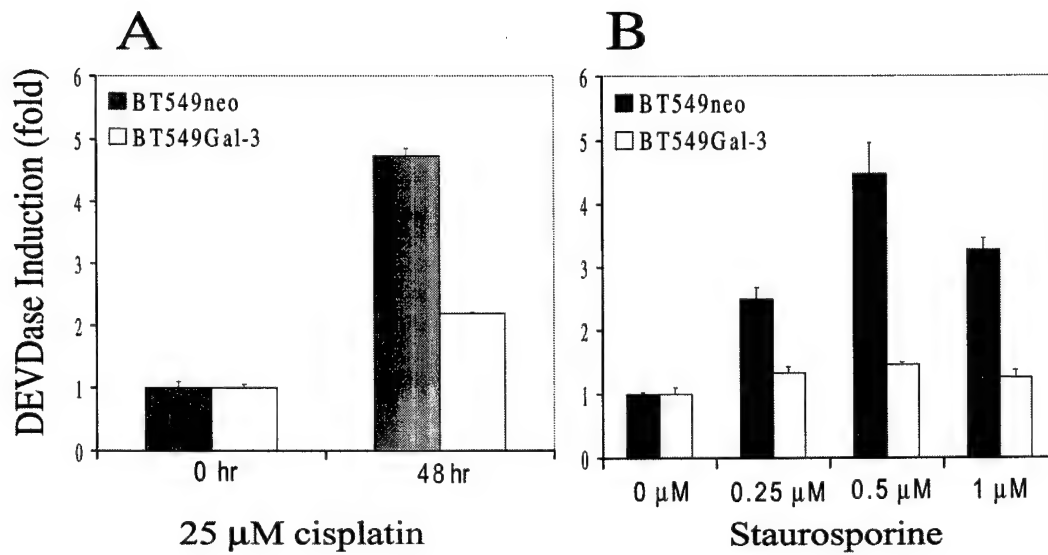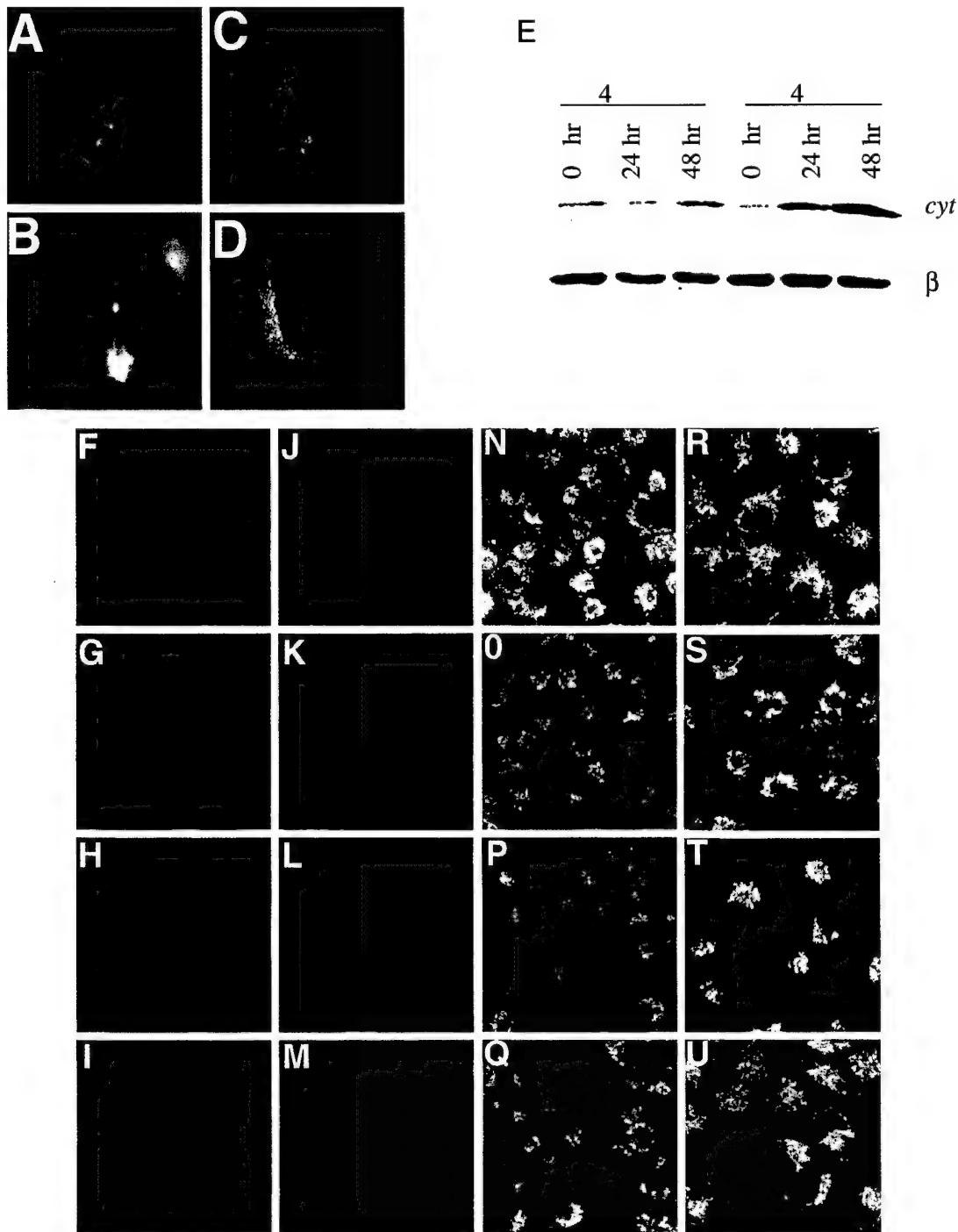
Figure 1. Galectin-3 expression in human breast epithelial cells downregulates DEVDase activity.
BT549neo and BT549Gal-3 cells grown in 35 mm dishes were treated with 25 μM cisplatin for 48 hrs (A) or staurosporine for 150 minutes (B). DEVDase activity was normalized per μg protein. Three independent experiments were performed and the error bars represent standard deviation of the mean of triplicates. The level of DEVDase activity in untreated cells was arbitrarily given as 1.
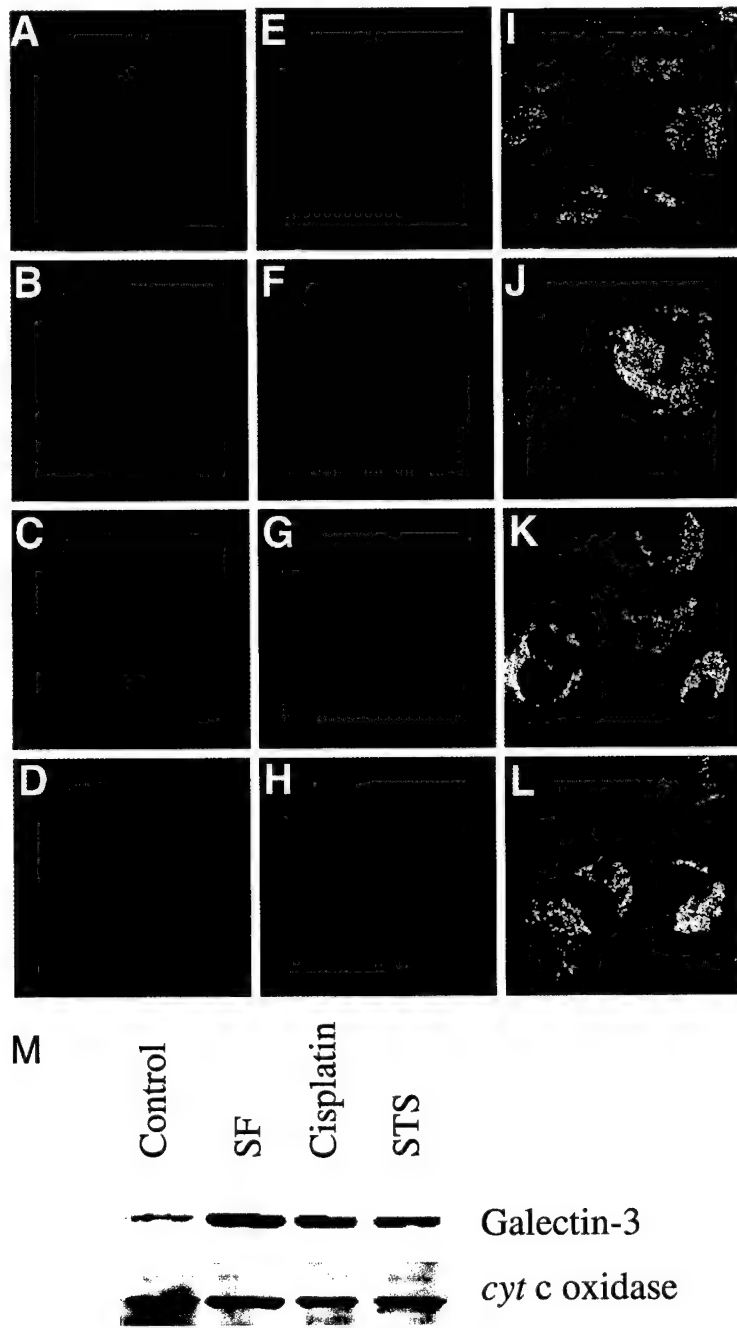
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Figure 2. Galectin-3 expression in human breast epithelial cells prevents mitochondrial damage and cytochrome c release. A-D, BT549neo (A,B), and BT549Gal-3(C,D) were cultured on cover slips and treated with 0 (A,C) or 25 μM cisplatin (B,D). After 36 hr, the cells were stained with a fluorescent probe for the mitochondrial membrane potential (MitoTracker Red). The fluorescence study was carried out with a Nikon Labophot microscope fitted with a digital video camera (Photometrics, Tucson, AZ). E, Immunoblot analysis of cytosolic cytochrome c was performed using cytosolic proteins prepared from BT549Gal-3 and BT549neo cells treated without or with 25 μM cisplatin for 24 hr or 48 hr. To confirm the equal loading of proteins in each lane, the same blot was reprobed with anti-β-actin antibody. F-U, BT549neo (F-I and N-Q) and BT549Gal-3 (J-M and R-U) were cultured on cover slips under no treatment (F,J,N,R), 25 μM cisplatin for 24 hrs (G,K,O,S), serum free media for 48 hrs (H,L,P,T)and 0.5 μM staurosporine for 150 min (I, M, Q, U). The mitochondria and cytochrome c were stained as described in materials and methods. F-M shows cytochrome c staining only, N-U are composite images of cytochrome c (green) and mitochondria (red), the yellow color indicating co-localization.

Figure 2. Galectin-3 expression in human breast epithelial cells prevents mitochondrial damage and cytochrome c release. A-D, BT549neo (A,B), and BT549Gal-3(C,D) were cultured on cover slips and treated with 0 (A,C) or 25 μM cisplatin (B,D). After 36 hr, the cells were stained with a fluorescent probe for the mitochondrial membrane potential (MitoTracker Red). The fluorescence study was carried out with a Nikon Labophot microscope fitted with a digital video camera (Photometrics, Tucson, AZ). E, Immunoblot analysis of cytosolic cytochrome c was performed using cytosolic proteins prepared from BT549Gal-3 and BT549neo cells treated without or with 25 μM cisplatin for 24 hr or 48 hr. To confirm the equal loading of proteins in each lane, the same blot was reprobed with anti-β-actin antibody. F-U, BT549neo (F-I and N-Q) and BT549Gal-3 (J-M and R-U) were cultured on cover slips under no treatment (F,J,N,R), 25 μM cisplatin for 24 hrs (G,K,O,S), serum free media for 48 hrs (H,L,P,T)and 0.5 μM staurosporine for 150 min (I, M, Q, U). The mitochondria and cytochrome c were stained as described in materials and methods. F-M shows cytochrome c staining only, N-U are composite images of cytochrome c (green) and mitochondria (red), the yellow color indicating co-localization.

Fig 3. Galectin-3 is redistributed onto the intracellular membranes following apoptotic stimuli. A-L: Control (A, E, I) and apoptotic BT549Gal-3 cells treated with 25 μM cisplatin for 24 hours (B,F,J), cultured in serum-free medium for 48 hr (C,G,K) and treated with 0.5 μM staurosporine for 150 minutes (D,H,L) were stained with MitoTracker Red and with anti-galectin-3 Ab/FITC conjugated 2nd Ab. Galectin-3 (green staining, A-D), mitochondria (red staining, E-H) and composite images (I-L) are shown. Yellow staining indicates co-localization of galectin-3 and mitochondria. M: Mitochondria were isolated from control and apoptotic BT549Gal-3 cells induced by culturing in serum-free medium for 48 hr (SF), 25 μM cisplatin treatment for 24 hours (Cisplatin) and 0.5 μM staurosporine treatment for 150 minutes (STS). The levels of the galectin-3 protein in the mitochondria were detected by immunoblot analysis (top panel). To confirm the equal loading of the mitochondrial proteins in each lane, the identical blot was probed with an anti-cytochrome c oxidase antibody (Molecular Probes, OR).
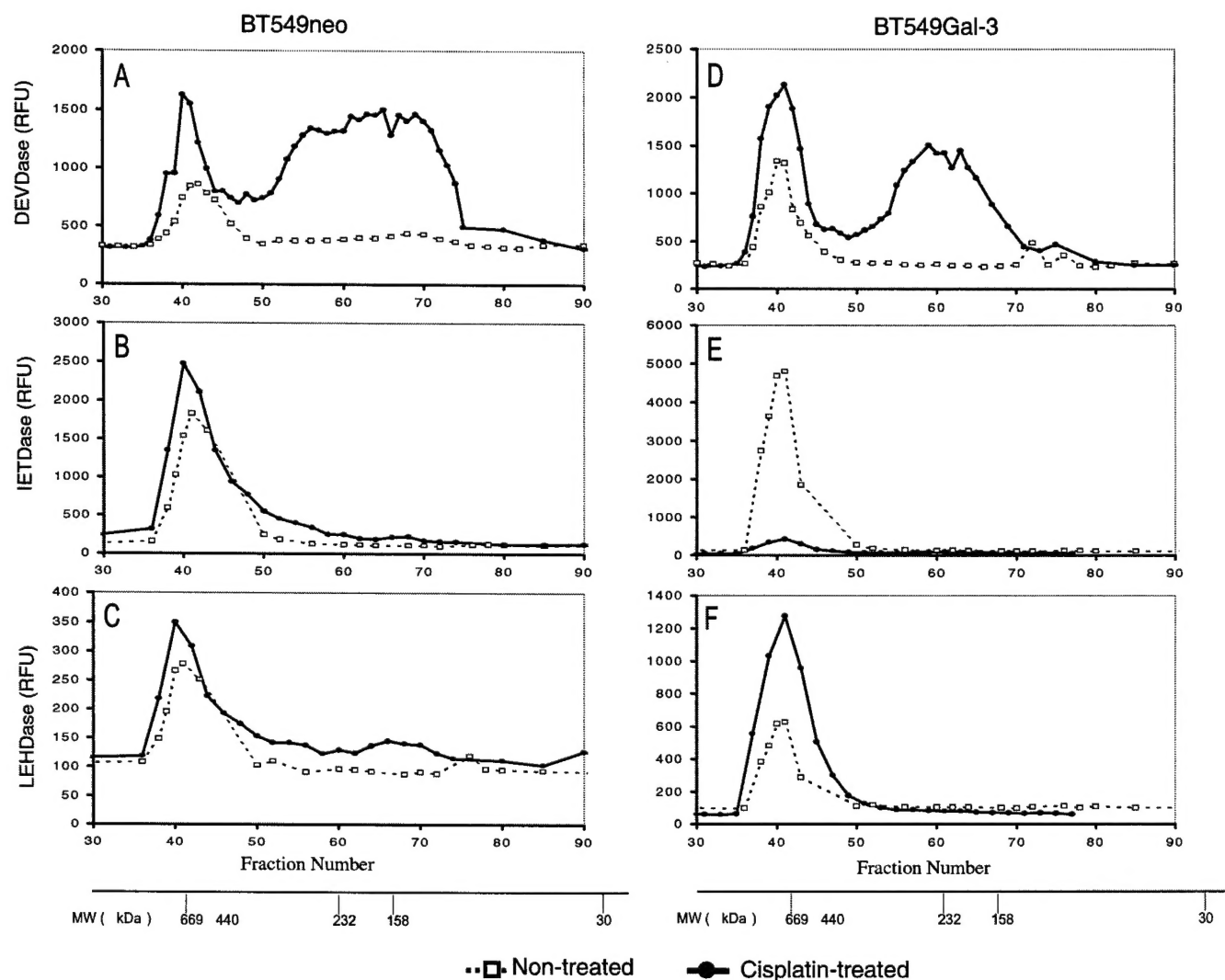
Figure 4. Caspase activity in the apoptosome and micro-apoptosome. BT549neo (A-C) and BT549Gal-3 (D-F) cells were treated without or with 25 μM cisplatin for 24 hr. Cell lysates were fractionated by size-exclusion chromatography. Fifty μl elutant of each 1 ml-fraction was assayed for DEVDase (A,D), IETDase (B,E)and LEHDase (C,F) activities. RFU represents relative fluorescence unit. Molecular weight was estimated using protein standards: thyroglobulin (MW 669,000), ferritin (MW 418,000), catalase (MW 206,000), and bovine serum albumin (MW 67,000).
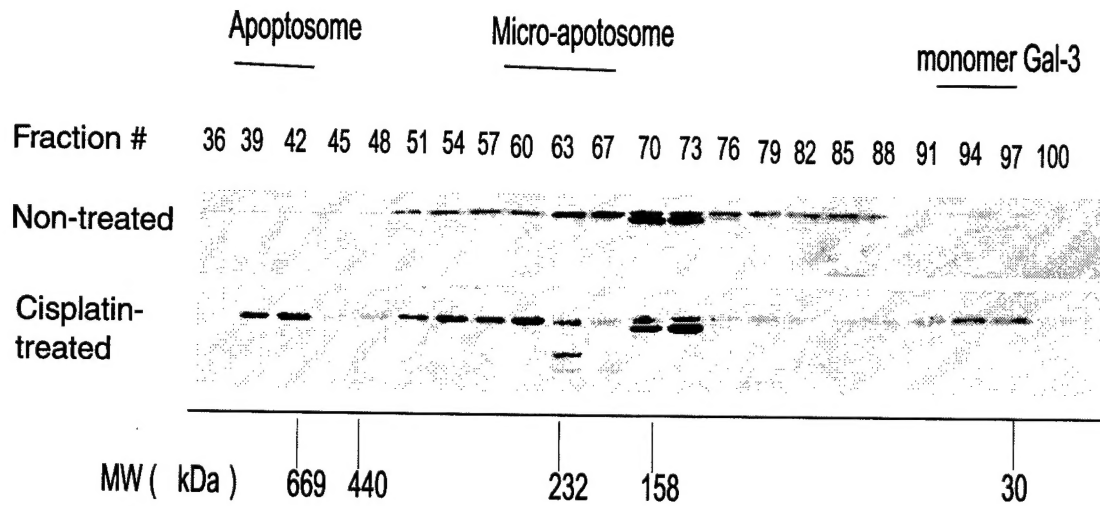
Figure 5. Galectin-3 is found in the apoptosome fractions. Fifty µl elutant of 1 ml-fraction (see Fig. 4) of untreated and cisplatin-treated BT549Gal-3 cell lysates were analyzed by SDS gel electrophoresis and immunoblot analysis using anti-galectin-3 antibody.
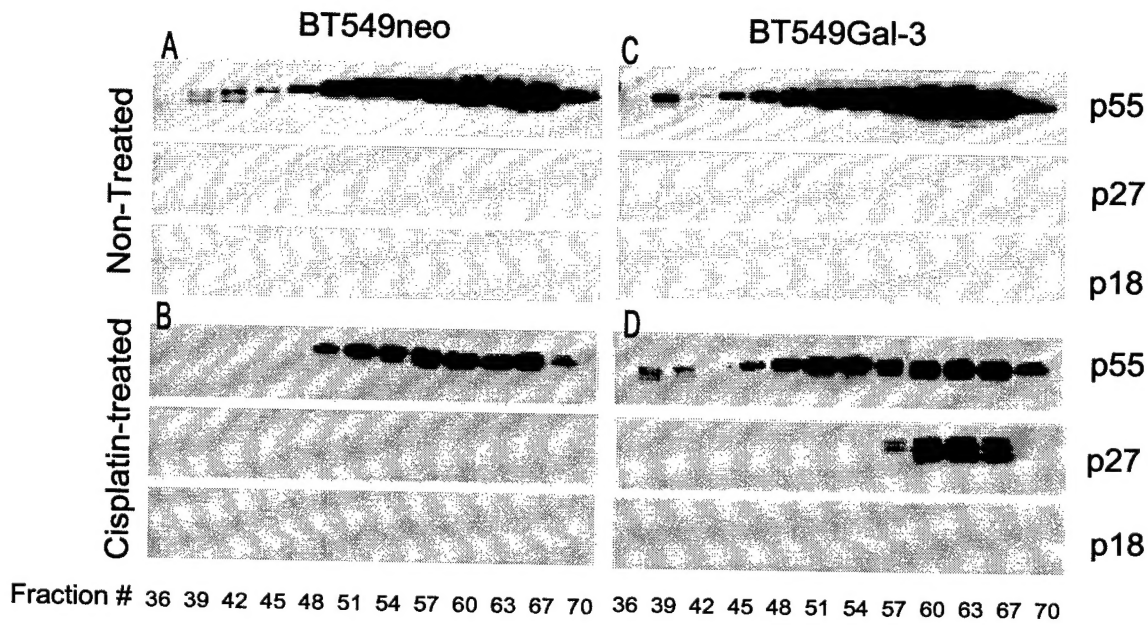
Figure 6. Accumulation of an enzymatically inactive intermediate form of caspase-8 in BT549Gal-3 cells. Fifty µl elutant of 1 ml-fraction (see Fig. 4) of untreated (A, C) and cisplatin-treated (B, D) BT549neo (A, B) and BT549Gal-3 (C, D) cell lysates were analyzed by SDS gel electrophoresis and immunoblot analysis using anti-caspase-8 antibody (Oncogene Inc. CA).

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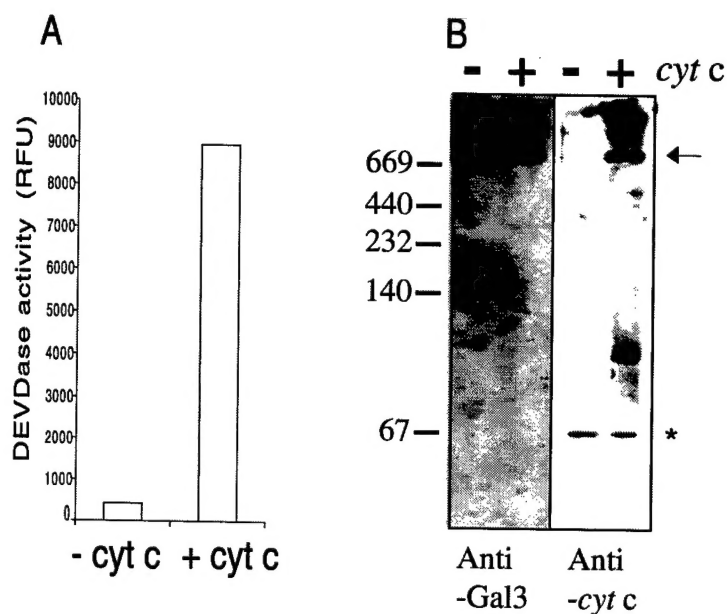
Figure 7. Galectin-3 is recruited into a ~700 kDa complex in cell-free lysates by cytochrome c. A. DEVDase activity was measured in 20 μg BT549Gal-3 lysates without or with 50 μg/ml cytochrome c at 37 °C for 30 minutes. B. BT549Gal-3 lysates (80 μg) were incubated without or with 50 μg/ml cytochrome c on ice for 30 minutes and resolved on a native gradient (4-12%) Tris-Glycine polyacryamide gel. After eletrophoresis, the proteins were denatured in 0.5% SDS and blotted onto nitrocellulose membrane. The blot was denatured in 0.1 % SDS, 0.8 % β-mercaptoethanol (v/v) and 62.5 mM Tris pH 6.8 at 70°C for 30 min, and subjected to immunoblot analysis using anti-galectin-3 and anti- cytochrome c antibodies. * indicates non-specific band.